



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : B01J 20/28, 20/32	A1	(11) International Publication Number: WO 00/20114 (43) International Publication Date: 13 April 2000 (13.04.00)
(21) International Application Number: PCT/GB99/03290 (22) International Filing Date: 5 October 1999 (05.10.99) (30) Priority Data: 9821783.9 6 October 1998 (06.10.98) GB (71) Applicant (for all designated States except US): BIOPROCESSING LTD. [GB/GB]; Unit 31, No. 1 Industrial Estate, Medomsley Road, Consett, Co. Durham DH8 6SZ (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): ANGUS, Katherine, Louise [GB/GB]; Bioprocessing Ltd., Unit 31, No. 1 Industrial Estate, Medomsley Road, Consett, Co. Durham DH8 6SZ (GB). HUTTON, David, Alan [GB/GB]; Bioprocessing Ltd., Unit 31, No. 1 Industrial Estate, Medomsley Road, Consett, Co. Durham DH8 6SZ (GB). NOEL, Robert, John [GB/GB]; Bioprocessing Ltd., Unit 31, No. 1 Industrial Estate, Medomsley Road, Consett, Co. Durham DH8 6SZ (GB). TAYLOR, Linda [GB/GB]; Bioprocessing Ltd., Unit 31, No. 1 Industrial Estate, Medomsley Road, Consett, Co. Durham DH8 6SZ (GB). (74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: ADSORBENT MEDIUM AND ITS USE IN PURIFYING DNA (57) Abstract An adsorbent medium comprises particles of a cellulosic sponge material carrying functional groups. This medium is especially useful for purifying DNA in an aqueous sample.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ADSORBENT MEDIUM AND ITS USE IN PURIFYING DNA

Field of the Invention

This invention relates to an adsorbent medium and to its use in retaining large molecular species, e.g. in the purification of DNA.

Background of the Invention

DEAE (diethylaminoethane) has been used to derivatise Sepharase or Sephadex beads to provide an anion exchange resin, having quaternary ammonium groups. This is available from Pharmacia as DEAE-streamline. It is used as an anticholesteremic.

Cellulosic sponge materials have been used in various forms as the basis of an adsorbent medium, typically after the introduction of ion-exchange groups. For example, GB-A-1226448 discloses the introduction of cross-linking residues into regenerated cellulose, together with or followed by the introduction of ion-exchange groups. The resultant medium can be used, packed in a column. Various physical forms are disclosed.

WO-A-9117830 discloses an adsorbent medium (HVFM) prepared by cross-linking a flexible hydrophilic sponge that contains fibrous, hydrophilic reinforcement, and introducing functional groups. The cross-linking is controlled so that the resulting sponge has a water retention value of 2 to 6. It is proposed that this medium is suitable for the isolation or separation of macromolecules such as proteins, while retaining mechanical strength. However, in addition to its low water retention value, this medium has poor flow properties.

A particular area in which an efficient, commercial process is required, for purifying samples, is in the preparation of pharmaceutical grade plasmid DNA for gene therapy. Although many processes have been developed for the large-scale production of recombinant plasmid DNA, e.g. using *E. coli* as the host cell, purification of the product is necessary before the functional gene can be used, i.e.

for expression in somatic tissues with the intention of selectively correcting or modulating disease conditions.

Purification of plasmid DNA has traditionally been on a very small scale for research purposes. Purification of plasmid DNA for preclinical toxicology, human clinical trials, and ultimately for an approved pharmaceutical indication, requires a process that reproducibly meets all the quality and regulatory standards and can be used to purify large quantities of the material economically.

10 The standard method for plasmid DNA purification by molecular biologists has been by caesium chloride/ethidium bromide ultracentrifugation. This method is unacceptable for the production of clinical materials, because it uses mutagenic reagents and is unscalable.

15 Alternative methods for the purification of plasmid DNA have been developed using a combination of chromatography techniques. Problems associated with such techniques have been low capacity of the adsorbents for plasmid DNA, denaturation and breakdown of the DNA molecule, and losses due to filtration of the feedstock containing plasmid DNA. Problems associated with filtration have been resolved by using the adsorbents in fluidised bed mode, but this requires specially designed hardware for large-scale applications.

25 Summary of the Invention

This invention is based on the discovery that an adsorbent medium comprising particles of a cellulose sponge material carrying functional groups, can be effectively used to retain species having molecular weight of at least one million Daltons, e.g. viruses and DNA. In particular, it has been found that an effective adsorbent can be prepared from readily-available sponge material, after removal of fibrous reinforcement.

35 The new porous matrix has high capacity for plasmid DNA, and can be used in a packed bed with unfiltered feedstock. It can be used in any type of chromatographic

technique, including ion-exchange, and also affinity and hydrophobic interaction.

Description of the Invention

5 The cellulosic sponge material, from which the novel medium is derived, is typically a naturally-occurring polymer such as cellulose or agarose.

Cellulosic sponge materials are commercially available, including hydrophilic, fibrous reinforcement. For the purposes of the present invention, this
10 reinforcement can be removed, or at least substantially removed. The water retention of the medium is generally higher than that described in WO-A-9117830, and is typically greater than 6 ml/g, e.g. up to 10 or 11 ml/g.

The material used in the invention is preferably
15 cross-linked. This may be achieved by known procedures. Similarly, the introduction of functional groups may be achieved in known manner, e.g. as described in WO-A-9117830. Suitable ion-exchange groups are known. It is preferred that the functional groups should bind DNA. They
20 may be derived from tertiary amines such as diethylaminoethane (DEAE), or quaternary amines.

The adsorbed medium of this invention may be made into particles of suitable size by any appropriate technique, e.g. chopping. The size of the particles may be
25 heterogeneous; it is not necessary to control its uniformity. The particles will usually be at least 0.1 mm in size, e.g. up to 10 mm or more; a preferred particle size is 0.5 or 1 to 10 mm.

In an illustrative embodiment of the invention, an
30 open structure fibrous polymer adsorbent (HVFM) has been developed for the purification of plasmid DNA. In order to demonstrate its suitability, the material was converted into a weak anion-exchanger (DEAE-HVFM) and used to purify plasmid DNA from unfiltered feedstock.

35 Particulate HVFM was derivatised, up to a level of 1933 μ moles per g dry weight, with diethylaminoethane (DEAE). The characteristics of particulate DEAE-HVFM for

the purification of plasmid DNA from crude feedstock were determined. The dynamic capacity of DEAE-HVFM for plasmid DNA was $>1500 \mu\text{g}, \text{ml}^{-1}$ and $1350 \mu\text{g}, \text{ml}^{-1}$ at $88 \text{ cm}, \text{h}^{-1}$ (30 column volumes per hour) and $175 \text{ cm}, \text{h}^{-1}$ (60 column volumes per hour), respectively.

Analysis of the purified plasmid DNA was carried out by various methods: it contained no detectable amount of RNase and was not damaged by this purification process. The pressure/flow properties of particulate DEAE-HVFM showed that a flow rate of 116 column volumes per hour can be achieved at a pressure of 2.2 bar, across a column having a bed height of 4 cm and which is 2.5 cm in diameter.

The water retention value of the particulate DEAE-HVFM is approximately 10 ml/g. A water retention value of 2 to 6 is quoted for HVFM with a uniformly distributed fibrous reinforcement (WO-A-9117830).

The following Examples illustrate the invention.

White HVFM Cloth (18 x 20cm) was washed in tap water at 50°C followed by RO H_2O and rolled dry. The HVFM was cut into pieces (approximately 2cm square) and the 'nylon' scrim reinforcement was removed. The HVFM pieces were covered with water in a plastic beaker and homogenised using a hand-held Kenwood Blender for approximately 2 minutes. Homogenised HVFM was poured into a Pharmacia XK-50 column and squeezed dry using the plunger of the column.

430ml H_2O and 3.5ml 1,3-dichloropropanol were mixed and added to the homogenised HVFM in a 500ml glass beaker and stirred gently to mix. The beaker was covered with non-PVC cling film (four sheets) and 1 layer of aluminium foil and incubated for 1h at 60°C in an oven. The reagents were poured off and the sponge washed and dried under vacuum suction and then by plunging in an XK-50 column.

55.4g DEAE was dissolved in 122ml RO H_2O and poured onto the cross-linked HVFM in a 500ml glass beaker and gently mixed. 250ml 5M NaOH was poured evenly onto the HVFM and mixed gently. The beaker was covered in 4 sheets of

non-PVC cling film and one sheet of aluminium foil and incubated for 1h. at 60°C in an oven. The reagents were poured off and the sponge dried under vacuum suction. Fresh solutions of 45% (w/v) DEAE and 5M NaOH were added to the sponge as above and incubated again at 60°C. The reagents were poured off and the sponge washed extensively under vacuum suction, followed by squeezing dry in an XK-50 column. This procedure gave HVFM derivatised with DEAE.

A 1ml Omni-fit column (ID 0.66cm) was prepared by hydrating approximately 1g wet weight DEAE-HVFM with 20mM Tris pH 7.5 and packing it into the column using the column plunger. The column was then equilibrated with 0.2M NaCl (adjusted to pH 11.0 with NaOH) at 0.5ml.min⁻¹. 35 ml 12mM HCl was then washed through the column at 0.5ml.min⁻¹. 1ml fractions were collected and the pH of each fraction measured. The column was then unpacked and the DEAE-HVFM dried to constant weight at 50°C in an oven. A graph of pH against mmoles of HCl applied to the column was plotted and the point at which the pH dropped sharply to approximately pH 3.0 was used to calculate the level to which the sponge had been derivatized with DEAE.

In summary, white sponge cloth that had been filleted of its reinforcing mesh and also homogenised was derivatized. The level of derivatization achieved was 1933µmoles DEAE per g dry weight.

The capacity of DEAE-HVFM (homogenised) was at least 5 times as much as that estimated for DEAE-streamline, i.e. 150-300µg.mg⁻¹. With increased capacity for plasmid DNA, it might be expected that the DEAE-HVFM may also have increased capacity for RNA and protein; therefore, the levels of these residuals between the two matrices was compared. No detectable RNAase, the predominant protein contaminant in the feedstock, was found in the 0.5M and 1.0M elutions of either matrix after NuPAGE electrophoresis and silver staining. RNAase could be detected in the breakthrough and the 20mM Tris washes from both matrices. Gel filtration on Sephacryl S-500 showed that low molecular

weight contaminants were present in the 1.0M elutions from each of the columns. SYBR green II staining of samples of the 1.0M elutions run on 1.0% agarose gels suggested that these contaminants are digested RNA, and the proportion of material in the plasmid DNA peak from the DEAE-HVFM column was greater than that from the DEAE-Streamline column (5.4% cf 1.7%), i.e. a three-fold increase in plasmid DNA purity relative to RNA.

Agarose gel electrophoresis of plasmid DNA eluted from both DEAE-HVFM and DEAE-streamline demonstrated that the plasmid was not damaged by binding and elution from the column. In contrast, silica-based matrices such as that found in the Plasmix purification kit nick the DNA and produce more of the open circular form.

The pressure/flow characteristics of particulate DEAE-HVFM were compared with those of DEAE-HVFM cloth with reinforcement and DEAE-HVFM 'natural' block. For all matrices, the flow rate increased with decreasing bed height. However, when the matrices were compared at a fixed bed height, the particulate DEAE-HVFM had better flow properties. For example, particulate DEAE-HVFM achieved a flow rate of 116 column volumes per hour at a pressure of 2.2 Bar, across a column of dimensions 4.0cm bed height and 2.5cm diameter; the flow rates for DEAE-HVFM reinforced cloth and DEAE-HVFM 'natural' block were 79 and 85 column volumes per hour respectively.

The DEAE-HVFM was also found to have better pressure/flow characteristics than Whatman Express ion-exchangers Q and DEAE.

CLAIMS

1. An adsorbent medium comprising particles of a cellulosic sponge material carrying functional groups.
2. A medium according to claim 1, wherein the sponge
5 material is free of fibrous reinforcement.
3. A medium according to claim 1 or claim 2, which has a water retention value of greater than 6 ml/g.
4. A medium according to any preceding claim, wherein the sponge material is a naturally-occurring polymer.
- 10 5. A medium according to claim 4, wherein the polymer is cellulose or agarose.
6. A medium according to any preceding claim, wherein the functional groups bind DNA.
7. A medium according to claim 6, wherein the functional
15 groups are derived from DEAE.
8. A medium according to any preceding claim, wherein the particles are 0.5 to 10 mm in size.
9. A medium according to any preceding claim, capable of retaining species having a molecular weight of at least one
20 million Daltons.
10. A medium according to any preceding claim, wherein the sponge material is cross-linked.
11. A method for purifying DNA in an aqueous sample, which comprises passing the sample through a medium according to
25 any preceding claim.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/03290

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 B01J20/28 B01J20/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 17830 A (BIO ISOLATES LTD) 28 November 1991 (1991-11-28) cited in the application	1,4,5,9, 10
Y	page 3, line 13 - line 15; claims 1,9,10 ---	6,7,11
Y	WO 94 11103 A (WILLIAMS) 26 May 1994 (1994-05-26) page 2, line 7 - line 13; claims 1,3,5,7,8 page 3, line 6 - line 14 ---	6,7,11
X	EP 0 451 706 A (SAKAI ENGINEERING) 16 October 1991 (1991-10-16) page 4, line 17 page 5, line 9 - line 11 ---	1,4,5
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 November 1999

Date of making of the international search report

08/12/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hilgenga, K

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/03290

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 154 676 A (D.T. JONES) 15 May 1979 (1979-05-15) column 2, line 44 column 2, line 25 column 4, line 24 - line 27 ---	1,4-7, 9-11
X	US 4 332 916 A (B.P. THILL) 1 June 1982 (1982-06-01) column 1, line 56 - line 60; claim 1 ---	1,2,4,5, 10
X	GB 1 226 448 A (TASMAN VACCINE LAB.) 31 March 1971 (1971-03-31) cited in the application page 1, line 82 page 3; claim 1; example 6 ---	1,4,5,10
X	US 5 162 404 A (RAINER NORMAN B) 10 November 1992 (1992-11-10) claim 1 ---	1,4,5,8
A	DATABASE WPI Section Ch, Week 199029 Derwent Publications Ltd., London, GB; Class A96, AN 1990-220679 XP002123920 & JP 02 149341 A (KANEGAFUCHI CHEM KK), 7 June 1990 (1990-06-07) abstract ---	1,4,5
A	DATABASE WPI Section Ch, Week 198646 Derwent Publications Ltd., London, GB; Class A96, AN 1986-303144 XP002123921 & JP 61 226059 A (AGENCY OF IND SCI & TECHNOLOGY), 7 October 1986 (1986-10-07) abstract ---	1,4,5
A	GB 914 421 A (SPONCEL LIMITED) page 2, line 7 - line 8 ---	1,4-7
A	EP 0 837 091 A (YAMAGUCHI) 22 April 1998 (1998-04-22) claim 1 -----	1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/03290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9117830 A	28-11-1991	DE 69113266 D	26-10-1995
		DE 69113266 T	24-10-1996
		DK 530258 T	05-02-1996
		EP 0530258 A	10-03-1993
		ES 2080316 T	01-02-1996
		US 5492723 A	20-02-1996
WO 9411103 A	26-05-1994	AU 5427394 A	08-06-1994
		EP 0621802 A	02-11-1994
EP 451706 A	16-10-1991	JP 3290443 A	20-12-1991
		AT 101813 T	15-03-1994
		CA 2039843 A,C	07-10-1991
		DE 69101225 D	31-03-1994
		DE 69101225 T	14-07-1994
		DK 451706 T	28-03-1994
		US 5298615 A	29-03-1994
US 4154676 A	15-05-1979	GB 1387265 A	12-03-1975
		AU 469879 B	26-02-1976
		AU 4475572 A	24-01-1974
		BE 786602 A	16-11-1972
		CA 975358 A	30-09-1975
		CH 606117 A	13-10-1978
		DE 2235902 A	01-02-1973
		ES 405084 A	01-07-1975
		FR 2147139 A	09-03-1973
		IE 36584 B	08-12-1976
		IL 39934 A	31-03-1976
		IT 963295 B	10-01-1974
		JP 48048583 A	10-07-1973
		JP 53015470 B	25-05-1978
		NL 7210142 A,B,	25-01-1973
		NO 137755 B	09-01-1978
		SE 408271 B	05-06-1979
		US 3905954 A	16-09-1975
		ZA 7205059 A	25-04-1973
US 4332916 A	01-06-1982	NONE	
GB 1226448 A	31-03-1971	BE 736062 A	16-12-1969
		DE 1935984 A	22-01-1970
		FR 2012985 A	27-03-1970
		NL 6910646 A	19-01-1970
		US 3573277 A	30-03-1971
US 5162404 A	10-11-1992	US 5002984 A	26-03-1991
		AU 635302 B	18-03-1993
		AU 6103490 A	21-02-1991
		CA 2023387 A	19-02-1991
		JP 3185024 A	13-08-1991
		US 5096946 A	17-03-1992
		US 5169883 A	08-12-1992
JP 2149341 A	07-06-1990	US 5064540 A	12-11-1991
		JP 1943236 C	23-06-1995
		JP 6067472 B	31-08-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/03290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 61226059 A	07-10-1986	JP 1026709 B JP 1540133 C	25-05-1989 31-01-1990
GB 914421 A		NONE	
EP 837091 A	22-04-1998	AU 2407397 A CA 2224879 A WO 9741169 A	19-11-1997 06-11-1997 06-11-1997